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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| (21) International Application Number: PCT/US95/01485 (22) International Filing Date: 2 February 1995 (02.02.95) (30) Priority Data: 08/190,436 2 February 1994 (02.02.94) US (71) Applicant: DEPARTMENT OF THE ARMY, US GOVERNMENT [US/US]; Office of Command Judge Advocate, HQ USAMRDC, Dept. of the Army, Fort Detrick, Frederick, MD 21702-5012 (US). (72) Inventors: CROSS, Alan, S.; 6810 Brookville Road, Chevy Chase, MD 20815 (US). WRIGHT, Daniel, G.; 2 Swallow Cave Road, Nahant, MA 01908 (US). GOMATOS, Peter, Suite 600, 1625 SE Third Avenue, Fort Lauderdale, FL 33316 (US). STAMATOS, Nicholas; 1330 New Hampshire Ave. NW #1010, Washington, DC 20036 (US). (74) Agents: HENDRICKS, Glenna et al.; 9669-A Main Street, P.O. Box 2509, Fairfax, VA 22031 (US). | | (81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> |
| (54) Title: USE OF SIALIC ACID OR ANTIBODIES TO SIALIDASE AS ANTI-INFECTIOUS AGENTS AND ANTI-INFLAMMATORY AGENTS (57) Abstract Sialic acid compounds are effective in treating various inflammatory diseases, to include rheumatoid arthritis and pneumonitis. | | |

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APPLICATION FOR UNITED STATES PATENT

Title: USE OF SIALIC ACID OR ANTIBODIES TO SIALIDASE AS
ANTI-INFECTIOUS AGENTS AND ANTI-INFLAMMATORY AGENTS

Field of the Invention:

5 This invention relates to the alteration of eukaryotic cell
surfaces by administration of sialidase inhibitors or sialic
acid. Background of the Invention:

Part of the inflammatory process is accomplished by the
circulating white blood cells, especially neutrophils, by
10 (1) slowing down at a site within the blood vessel where an
inflammatory response is developing, (2) adhering to the
endothelial cells adjacent to the site of inflammation,
(3) exiting the intravascular space through the lining of the
blood vessels and (4) migrating to the inflammatory site within
15 the tissues. In order to achieve this complex series of steps,
the neutrophil must bind to the endothelial cells lining the
vessels and then "unbind" so that it can continue on into the
tissue. It is known that sialic acid residues on glycocon-
jugates of the cells are important in this initial binding.

20 The term "sialic acid" denotes members of a family
comprising natural derivatives of neuraminic acid, an acid amino
pyranose with 9 carbon atoms. In nature, the amino group is
substituted either with an acetyl or glycolyl residue. The
hydroxy groups may be methylated or esterified with groups such
25 as acetyl, lactyl, sulfate, or phosphate groups. Multiple
substitutions are common.

Sialic acids are a phylogenetically conserved family.
These amino sugars are conjugated to protein and lipid moieties
on the surface of mammalian cells and are potent modulators of
30 biologic behavior. There is substantial evidence that sialic
acids are structural determinants of important cell-to-cell
interactions and cellular functions such as adhesiveness. There
is considerable evidence that sialic acid residues protect
molecules in circulation from recognition, clearance or
35 degradation and that they regulate complement deposition on cell
surfaces. Sialic acid residues also modulate attachment of
microbial toxins as well as parasites to these cell surfaces.

The cleavage of the sialic acid by sialidases or neuraminidases from the glycoconjugates results in decreased rigidity of the cell surface, thereby facilitating cell motility, and effects cell-to-cell interactions such as adhesiveness and metastatic potential. Sialidases or neuraminidases are produced by many microbes and by mammalian cells. Whereas the presence of endogenous sialidase of mammalian cells has been well described, its role has best been studied primarily in a clinically heterogenous group of inherited disorders designated as sialidoses, wherein an abnormal amount of sialic acid accumulates in tissues of patients resulting in neurologic defects and premature death.

Endogenous sialidase in phagocytes has previously been described. It has been found that, upon activation such as may occur during infection or inflammation, this enzyme is translocated to the cell surface from sites within the cell (Cross and Wright, Journal of Clinical Investigation, Inc., 88 (December, 1991) pp 2067-2076). The result of this mobilization is the removal of significant quantities of cell-associated sialic acid from glycoconjugates on cell surfaces. Desialylation of resting cells by microbial neuraminidase or of activated cells by mobilization of endogenous sialidases remove negative electric charges from cell surfaces and alters the biologic behavior of these cells to that typically observed during inflammation. Activation of cells in the presence of known sialidase inhibitors such as exogenous sialic acid prevents the desialylation and lowers cell adherence.

Infection of mammalian cells by HIV is known to be facilitated by activation of its cellular target. The critical events in the multi-step process of cellular activation that facilitates infection with HIV have not been identified. It has been shown that increased expression of endogenous sialidase follows activation of T lymphocytes by lectins and it has been suggested that this increase may play a role in the differentiation and maturation of these cells. Sialidase-treated peripheral blood mononuclear cells (PBMCs) support growth of HIV-1 in the absence of lectin activation. Treatment of PBMCs with

sialidase or lectin (phytohemagglutinin) results in hyposialylation of the PBMC.

Specific inhibitors of sialidase activity have been used in vivo in mice to decrease mutual adhesion of blood platelets and to inhibit accumulation of leucocytes in microvascular beds that had been laser-irradiated. (Gorog, et al, Br. J. exp. Path. 61 (1980), 490).

Various sialidase inhibitors have previously been tested. Kumar, et al. (Carbohydrate Research, 94 (1981) 123-130) disclosed a method of synthesizing various neuraminic acids. Nohle, et al. (J. Biochem, 126 (1982) 543-548) discloses methods of synthesis of 2-deoxy-2,3-dehydro-N-acetylneuraminic acid and 2-deoxy-2,3-dehydroneuraminic acid and discusses the oral administration and secretion of the sialic acids. No method of using these inhibitors for anti-infective or anti-inflammatory use are taught therein. Nagai, et al. (Biochemical and Biophysical Research Communications, Vol 163, No. 1 (1989) and Miyaichi, et al. (Shoyakugaku Zasshi, 42 (3)(1988) 216-219) disclose use of a natural product from the leaf of Scutellaria baicalensis as an inhibitor of mouse liver sialidase, but its application to the treatment of inflammation is not discussed.

Summary of the Invention:

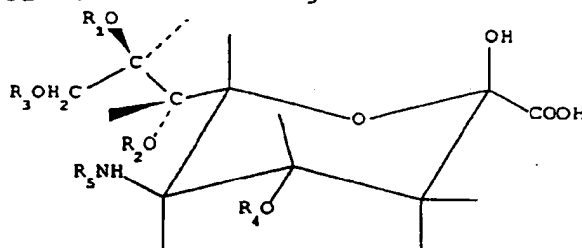
This invention provides a means of treating inflammatory conditions and of intervening in infectious processes in instances where pathogenicity of the disease-inducing organism is increased by microbial surface interaction with host cells. Sialic acids may be delivered at dosage of .1 to 10 mg/kg intravenously 4-6 time a day. For humans, dosage forms would contain 5 to 1000 mg of sialic acid or sialic acid analogue. For example, presence of 2,3-dehydro-2-desoxy-N-acetyl neuraminic acid (NANA) prevented desialylation of peripheral blood lymphocytes and ability of the cells to support infection by HIV. A second aspect of the invention relates to inhibition of sialidases by administration of antibodies which inhibit the action of sialidases.

Detailed Description of the Invention:

Sialic acids such as NANA have the ability to prevent

hyposialylation of cells by competitive inhibition of the endogenous The desialylation of cells is shown to increase adhesive properties of the cells and to render cells more susceptible to invasion by infectious organisms, particularly HIV. Cellular hyposialylation also accompanies inflammation. The sialidase inhibitors used as disclosed herein are effective in treating and/or in avoiding inflammation. It is possible that for the cell to "unbind" it must de-adhere or "unbind" in a manner that might involve the cleavage of sialic acid residues. The enzyme that would accomplish this cleavage is sialidase. It is reasonable to believe that inhibition of this enzymatic step results in a decreased ability of neutrophils to leave the intravascular space and migrate to inflammatory sites within tissue. Thus, any agent that inhibits sialidase can act as an anti-inflammatory agent.

Sialic acid and its analogues are of the formula:



wherein $R_{1,2,3}$, and 4 may be H, alkyl, SO_3H , PO_3H_2 or CO-alkyl, wherein alkyl has 1-4 carbons and may be substituted with OH and R_5 is CO-alkyl of 1-4 carbons which may be substituted with OH, with acetyl and glycolyl being preferred at R_5 .

MATERIALS AND METHODS:

Isolation and Culture of PBMCs:

Peripheral blood mononuclear cells were isolated from whole blood of HIV-1- and hepatitis B-seronegative donors by Ficoll-Paque (Pharmacia, Piscataway, New Jersey) density gradient centrifugation and were grown in RPMI 1640 medium (GIBCO, Grand Island, New York) containing 10% purified, delectinized human IL-2 (Advanced Biotechnologies, Inc., Columbia, Maryland) and 15% heat-inactivated fetal bovine serum (GIBCO). Cells were maintained at 37°C on CO_2 -humidified incubator for 24 to 72

hours prior to treatment with phytohemagglutinin (PHA) or neuraminidase, respectively.

Pretreatment of PBMCs with PHA or neuraminidase:

Cells at 10^7 cells/ml were treated with PHA at 5 μ g/ml for 30 minutes, were diluted 5-fold by addition of medium and were maintained in media containing PHA at 1 μ g/ml. After 48 hours, cells were removed from the PHA-containing medium by centrifugation prior to infection with HIV-1. Alternatively, after 72 hours in culture, untreated cells were collected and resuspended at 10^7 cells/ml for treatment at 37°C for 30 minutes with 1 U/ml microbial neuraminidase, unless other treatment conditions were indicated. Cells were removed from the enzyme by centrifugation and were washed once prior to infection. Macrophage-depleted cultures were prepared for treatment with PHA or NANase by collecting PBMCs which had not adhered to culture flasks after 24 or 72 hours, respectively. Cells exposed to PHA at 24 hours for stimulation during the subsequent 48 hours to provide 72 hours from seeding of cells to infection. Cells treated with sialidase were treated with enzyme 72 hours after seeding (immediately before infection). After 72 hours, PHA-or-sialidase-(NANase) treated cells were seeded in 96 well microtiter plates at 2.5×10^5 cells/well and were infected with HIV-1. Alternatively, infected cells were maintained in 75 cc flasks at 1×10^5 cells/ml.

HIV infection of PBMCs:

HTLV-IIIIB/H9 strain HIV adapted to T cell lines were used to infect the PBMCs in the wells of microtiter plates at 50, 5, 1 or 0.5 TCID₅₀ of HIV-1 in final volume of 0.25 ml in media containing POLYBRENE^R (Sigma) at 1 μ g/ml. (When infected cells were maintained in flasks, 0.8×10^7 cells were infected with 1.6×10^3 , 1.6×10^2 , and 1.6×10 TCID₅₀ in a volume of 2 ml for 2 hours at 37°C to remove unadsorbed virus, cells were centrifuged, washed once, and seeded in 75 cc flasks at 1×10^6 cells/ml.) In both the microtiter plates and in the flasks, cells were incubated at 37°C for 21 days. One half volume of media was removed one day post infection and every 2 days thereafter to be replaced with an equal volume of fresh medium.

Levels of p24 Ag in culture fluids were determined by ELISA (Coulter Immunology, Hialeah, Florida).

Determination of total cell-associated sialic acid:

5 Cells treated with PHA, NANase or untreated cells were washed three times in cold RPMI before exposure of pelleted cells to 0.1 M H_2SO_4 at 80°C for one hour to release cell-associated sialic acid. Free sialic acid was measured by the Warren Assay.

Determination of cellular sialidase activity:

10 PBMCs in RPMI 1640 medium containing 10% fetal bovine serum and 4 U/ml of recombinant human IL-2 (Boehringer Mannheim, Indianapolis, Indiana) were incubated at 37°C for 48 hours alone or with PHA (7.5 $\mu g/ml$). After 48 hours, cells were reisolated in a density gradient of Lymphocyte Separation Medium and were
15 washed twice. After resuspension of cells in RPMI 1640 at 80×10^6 cells/ml, sialidase activity was assayed by incubating intact cells with neuraminyl-lactose (Sigma) 1mg/ml at 37°C for 2 hours. Sialidase activity was determined by correlating the amount of sialic acid released from intact cells to a standard
20 curve generated by determination of sialic acid released from neuraminyl lactose by 10 mU/ml NANase.

Results:

Treatment of cells with sialidases rendered the peripheral blood mononuclear cells susceptible to infection with HIV, while
25 administration of sialic acids counteracted the effects of sialidase. (See Table I.)

Table I:

Susceptibility of pretreated PBMC's in neuraminidase and PHA:

| Experiment | HIV-1 inoculum (TCID ₅₀ /2.5x10 ⁵ cells) | <u>p24 Ag (ng/ml) released</u> <u>from cells pretreated with:</u> | | |
|------------|---|--|-----|--------|
| | | None | PHA | NANase |
| 5 | #1 | 50 | 32 | 73 |
| | | 5 | 7 | 72 |
| | | 1 | 0 | 69 |
| 10 | | | | 26 |
| 15 | #2 | 59 | 12 | ND |
| | | 5 | 0 | ND |
| | | 0.5 | 0 | ND |
| 20 | | | | 15 |
| | | | | 16 |
| | | | | 12 |
| 25 | #3 | 0.5 | 0 | 5 |
| | | | | 50 |
| | | | | |

It has also been found that the effect of sialidases can be counteracted by administration of competitive inhibitors of sialidases, specifically by administration of analogues of sialic acid. Neuraminic acid (NeuAc) and 2,3-dehydro-2-desoxy-N-acetyl-neuraminic acid (NeuAc2en) were tested as competitive inhibitors of neuraminidases. Growth of HIV was not detected in cells which had been pretreated with NANase in the presence of 250 μ M NeuAc or NeuAc2en. Hence, presence of the competitive inhibitors of the NANase protected cells from vulnerability to the HIV-1. (See Table II.)

Table II:

Protective effect of sialic acid analogues on growth of HIV:

| | Conditions of NANase retreatment of PBMC's | p24 Ag (ng/ml) released | |
|----|---|-------------------------|--------|
| | | day 12 | day 22 |
| 5 | 1 U/ml, 240 min | 54 | 56 |
| | 1 U/ml, 30 min | 59 | 56 |
| 10 | 0.1 U/ml, 30 min | 0 | 0 |
| | 1 U/ml + NeuAc (250 μ M), 30 min | 0 | 0 |
| 15 | 1 U/ml + NeuAc2en (250 μ M), 30 min | 0 | 0 |
| | Heat-inactivated NANase, 30 min | 0 | 0 |

20 Sialic acid analogues are effective at dosages of about .1 to 10 mg/Kg for 4 to 6 hours. Administration intravenously of the sialic acid analogue can be used to effectively protect cells from infection with HIV. By methods of the invention, the patient with HIV may be placed on a regimen to protect the cells

25 through one replacement cycle for the PBMC's.

 Synthesis of viral DNA is initiated following infection of both activated and quiescent primary lymphocytes, but HIV genomic RNA is not transcribed completely for full-length double-stranded DNA in quiescent PBMC. HIV can remain latent

30 in quiescent cells for an extended period of time, but if mitogenic stimulation occurs within 4-8 days of infection, synthesis of viral DNA is completed and growth of virus occurs. Hence, administration of sialic acid analogues can be used to protect cells during early stages of infection (the period

35 immediately after exposure to HIV) from completion of the viral growth cycle.

Modulation of Inflammatory Response

The injection of thioglycollate into the peritoneum of animals is a well-established experimental technique for eliciting an inflammatory response. Therefore, mice were pre-
5 treated intravenously with either a competitive inhibitor of sialidase (sialic acid was used) or with a polyclonal rabbit anti-sialidase antisera in an attempt to have inhibitors of sialidase circulating in the blood at the time of initiation of the inflammatory process with thioglycollate. It was reasoned
10 that if these agents were effective in inhibiting sialidase and if sialidase were important in the inflammatory response, a decreased amount of cells migrating into the peritoneal site of inflammation would be seen.

Preventive effects in mice:

15 Three groups of mice were studied for response to injections of thioglycollate in amounts sufficient to cause an inflammatory response. All groups were pretreated:

Group 1: Mice were pretreated intravenously with normal rabbit serum.

20 **Group 2:** Mice were pretreated intravenously with rabbit antisera containing increased levels of antibodies to sialidase.

Group 3: Mice were pretreated intravenously with sialic acid.

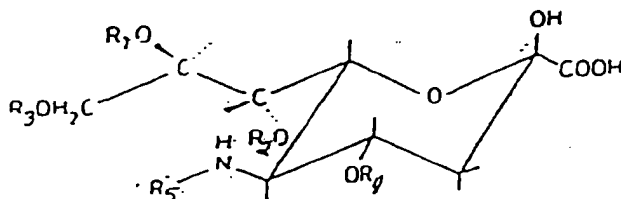
Results:

25 Mice in groups 2 and 3 showed less inflammatory response than the mice treated with normal rabbit serum. Hence, it was seen that both antibodies to sialidase and sialic acid showed anti-inflammatory effects.

Sialic acid and sialic acid analogues may be given intravenously or into the site of inflammation. For example, the active agents may be administered into, for example joints or the peritoneal cavity.

Claims

1. A composition of matter in dosage form comprising an anti-inflammatory effective amount of sialic acid or a sialic acid analogue of the formula:



wherein $R_{1,2,3}$, and 4 may be H, alkyl, SO_3H , PO_3H_2 or CO-alkyl, wherein alkyl has 1-4 carbons and may be substituted with OH, and R_5 is CO-alkyl of 1-4 carbons which may be substituted with OH, with acetyl and glycolyl being preferred at R_5 , in a sterile pharmaceutically acceptable carrier-contained within a sealed container.

2. A composition of claim 1 wherein the dosage of active agent is 5-1000 mg.

3. A composition of claim 1 wherein the anti-inflammatory agent is the sialic acid analogue 2,3-dehydro-2-desoxy-N-acetyl-neuraminic acid.

4. A composition of claim 1 wherein the anti-inflammatory agent is sialic acid.

5. A method of treating inflammation by administration of an anti-inflammatory pharmaceutical composition of claim 1.
- 5 6. A method of claim 5 wherein the anti-inflammatory composition is administered locally to a site of inflammation.
7. A method of claim 5 wherein the anti-inflammatory composition is administered intravenously.
- 10 8. A method of claim 6 wherein the anti-inflammatory composition is administered by injection into a joint.
9. A method of protecting peripheral blood morphonuclear cells from infection with HIV by administration of sialic acid or an analogue thereof at a dosage sufficient to obtain an HIV infection-inhibiting blood level of the sialic acid or sialic acid analogue in a pharmaceutically acceptable carrier every 4 to 6 hours intravenously.
- 15 10. A method of claim 9 wherein the dosage administered is .1 to 10 mg/Kg.
- 20 11. A method of claim 10 wherein the sialic acid analogue administered is 2,3-dehydro-2-desoxy-N-acetyl-neuraminic acid.
- 25

12. A method of claim 10 wherein the active agent is neuraminic acid.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/01485

| A. CLASSIFICATION OF SUBJECT MATTER IPC(6) : A61K, 31/35 US CL : 514/459 According to International Patent Classification (IPC) or to both national classification and IPC | | |
|---|--|--|
| B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 514/459 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) STN, compounds and anti-inflammatory methods of use. | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | |
| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| Y | Chemical Abstracts, volume 120, no. 12, issued 1994, Ippolito et al, "Immunosuppressive and tolerogenic oligosaccharide derivatives", see page 121, column 1-2, abstract no. 261339x, PCT Int Appl. WO92/22,301, see entire document. | 1-8 |
| Y | Eur. J. Biochem., Volume 126, issued 1982, Nohle et al, "Uptake, Metabolism and Excretion of Intravenously Administered, Double-Labeled N-Glycoloylneuraminic Acid and Single-Labeled 2-Deoxy-2,3-dehydro-N-acetylneuraminic Acid in Mouse and Rat", pages 543-548, see entire document. | 1-8 |
| <input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex. | | |
| * Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "Z" document member of the same patent family | | |
| Date of the actual completion of the international search 30 MARCH 1995 | | Date of mailing of the international search report 18 MAY 1995 |
| Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230 | | Authorized officer RUSSEL TRAVERS Telephone No. (703) 305-1235 |

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

1. International application No.
PCT/US95/01485

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos. 1-8

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/01485

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

Group I, claims 1-8 directed to a anti-inflammatory sialic acid compositions and methods for using such compositions against inflammations.

Group II, claims 9-12 directed to a method of protecting blood cells from HIV infections with sialic acid compositions.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The additional method of group II is properly grouped separately from that of group I pursuant to 37 CFR 1.475(d).